A CYCLIC PATHWAY FOR THE BACTERIAL DISSIMILATION OF 2,3-BUTANEDIOL, ACETYLMETHYLCARBINOL AND DIACETYL

II. The Synthesis of Diacetylmethylcarbinol from Diacetyl, a New Diphosphothiamin Catalyzed Reaction[1]

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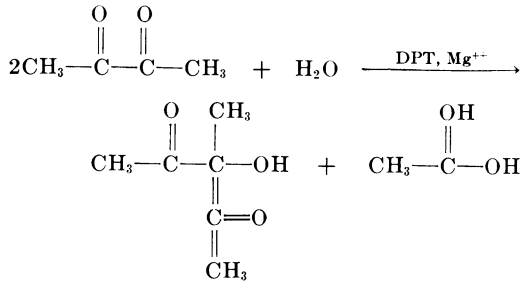
It has been demonstrated (Juni and Heym, 1956*a*) that microorganisms capable of using either 2,3-butanediol or acetylmethylcarbinol (AMC) as the sole source of carbon and energy for growth develop a cyclic pathway that results in the conversion of these compounds to acetic acid. Evidence has been presented for the ocurrence in these organisms of enzymes that oxidize 2,3-butanediol to acetylmethylcarbinol followed by the oxidation of the latter compound to diacetyl. Diacetyl then undergoes a non-oxidative diphosphothiamin (DPT) dependent acyloin condensation to form diacetylmethylcarbinol (DAMC) and acetic acid as indicated:



Once formed diacetylmethylcarbinol is further metabolized to acetylbutanediol (ABD) which, in turn, is hydrolyzed to 2,3-butanediol and acetic acid. These last two reactions are now being investigated and will be considered in detail in future reports.

The present paper is concerned with the purification and properties of the enzyme system that catalyzes the synthesis of diacetylmethylcarbinol and acetic acid from diacetyl.

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MATERIALS AND METHODS

Preparation of crude enzyme extract. Cells of an organism tentatively identified as *Micrococcus ureae* were grown and harvested as described previously (Juni and Heym, 1956*a*). Cell-free extracts were prepared by suspending the wet packed cells, obtained by centrifugation in a Sharples supercentrifuge, in five times their volume of distilled water and subjecting 60-ml aliquots to sonic vibration for 30 min with a Raytheon 10-kc oscillator. The resulting suspension was then centrifuged at 25,000 × G for 30 min at 5 C and the precipitate discarded.

Chemicals and chemical assays. The assays used for acetic acid, diacetyl, and diacetylmethylcarbinol have already been described (Juni and Heym, 1956*a*). In addition to these methods a new colorimetric procedure for determining small quantities of diacetylmethylcarbinol quantitatively has been devised (Juni and Heym, 1957) and is now used as the method of choice. During enzyme purification protein was determined either by the colorimetric method of Lowry *et al.* (1951) or by the optical method of Warburg and Christian (1941). The determination of protein by measuring the turbidity produced in the presence of trichloracetic acid (Stadtman *et al.*, 1951) was found to be unsatisfactory since purification of the enzyme system resulted in protein solutions that no longer react with this reagent. Alumina C γ was prepared according to Willstätter and Kraut (1923). "Amphojel," a commercial preparation of aluminum hydroxide gel, was obtained from Wyeth Inc. Diphosphothiamin and protamine sulfate were obtained from Nutritional Biochemicals Corp. The dimerization of diacetyl was followed by determining the residual unpolymerized diacetyl according to Westerfeld (1945). Diace-

tylmethylcarbinol was synthesized as previously described (Juni and Heym, 1956*b*). Enzymatically formed diacetylmethylcarbinol may be prepared in large quantities using the enzyme fractions described below (Juni and Heym, 1957). Since phosphate interferes with the colorimetric method for the determination of diacetylmethylcarbinol this compound must first be removed quantitatively from reaction mixtures containing phosphate buffers (Juni and Heym, 1957).

Enzyme assay methods. Acetic acid formation may be followed manometrically by measuring the initial rate of CO_2 evolution from bicarbonate buffer in the conventional Warburg apparatus at 30 C, with 100 per cent CO_2 as the gas phase. Suitable precautions concerning the volatility of diacetyl during gassing should be observed (Juni and Heym, 1956*a*).

A simple assay procedure has been devised (Juni and Heym, 1957) making use of the fact that diacetylmethylcarbinol reduces molybdate anion to the so-called molybdenum blue. The following assay mixture should be freshly prepared each day and kept in an ice bath to avoid loss of diacetyl due to volatilization: 2.0 ml of 0.5 M sodium maleate buffer, pH 6.4, 0.6 ml of a diphosphothiamin solution containing 0.5 mg of DPT per ml, 0.6 ml of 0.5 per cent $MgSO_4$, 0.1 ml of 1.0 M diacetyl, and 4.7 ml of distilled water. To a colorimeter tube is added 0.4 ml of the above assay mixture and the solution temperature equilibrated in a 30 C water bath for several min. The reaction is initiated by adding 0.1 ml of a suitably diluted enzyme fraction (usually 1:10 for the crude enzyme), mixing thoroughly, and incubating at 30 C for 10 min. To stop the reaction 1.0 ml of acid-molybdate reagent (Juni and Heym, 1957) is added, followed by 4.5 ml of distilled water. The color is permitted to develop for the required length of time for this test and read in a Klett colorimeter at 660 mμ (Juni and Heym, 1957). The amount of phosphate present in the diluted enzyme fraction is not sufficient to interfere in the color-forming reaction. A colorimeter blank is prepared by addition of the acid-molybdate reagent to 0.4 ml of assay mixture followed by 0.1 ml of the diluted enzyme solution. Activity is defined as μmoles of diacetylmethylcarbinol formed in 10 min per ml of enzyme. Specific activity is defined as μmoles of diacetylmethylcarbinol formed in 10 min by 1.0 mg of protein using the above assay procedure.

RESULTS

Stoichiometry of the reaction. Crude sonic extracts generally contain all the enzymes of the 2,3-butanediol cycle (Juni and Heym, 1956*a*). Addition of any of the intermediates of the cycle, except 2,3-butanediol, results in the conversion of the compound to 2,3-butanediol and acetic acid (Juni and Heym, 1956*a*). It has been observed, however, that high concentrations of diacetyl irreversibly inhibit several of the other reactions of the system and permit a relatively quantitative conversion to diacetylmethylcarbinol and acetic acid (table 1). Purification of the enzyme resulted in a fraction that was no longer capable of further metabolizing diacetylmethylcarbinol even when diacetyl was present in low concentration. The results obtained with such a fraction are shown in table 1. Studies with purified enzymes gave stoichiometric balances for the cases where the reaction proceeded either partially or to completion.

Aubert and Millet (1953) have reported the presence of a diacetyl mutase enzyme system (Green *et al.*, 1947) in extracts of *Neisseria winogradskyi* grown with 2,3-butanediol as the source of carbon and energy. Our studies (Juni and Heym, 1956*a*), however, have shown that several different genera of bacteria all carry out the same reactions of the 2,3-butanediol cycle and convert diacetyl to diacetylmethylcarbinol and acetic acid, there being no evidence for a diacetyl mutase reaction. Using diacetylmethylcarbinol as the substrate for partially fractionated extracts

TABLE 1

Balances for the anaerobic breakdown of diacetyl by cell-free extracts

Substrate and Products	Crude Enzyme		Purified Enzyme	
	Theoretical	Found	Theoretical	Found
	μmoles	*μmoles*	*μmoles*	*μmoles*
Diacetyl	141	—	25.4	—
DAMC	70.5	70.5	12.7	12.1
Acetic acid	70.5	66.0	12.7	12.1

$NaHCO_3$, 100 μmoles; diphosphothiamin, 50 μmoles; $MgSO_4$, 0.1 ml of a 0.5 per cent solution; enzyme, 0.5 ml; diacetyl, as indicated; gas phase, 100 per cent CO_2: final vol, 1.6 ml; incubation at 30 C.

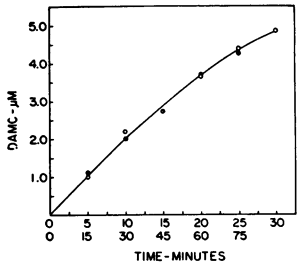

Figure 1. Diacetylmethylcarbinol formation as a function of time and enzyme concentration. Each tube contained the following: potassium phosphate buffer, 100 μmoles; pH 6.4; diphosphothiamin, 50 μg; $MgSO_4$, 0.1 ml of a 0.5 per cent solution; diacetyl, 50 μmoles; enzyme, 0.06 or 0.02 ml; final vol, 1.6 ml; gas phase air. The incubation temperature was 30 C. The reaction was stopped by acidifying samples at 5-min intervals when 0.06 ml of enzyme was used (⊙), and at 15-min intervals with 0.02 ml of enzyme (●).

it has been shown in our laboratory that there is sufficient oxidizable endogenous material in the extracts to reduce diacetylmethylcarbinol to acetylbutanediol in the presence of diacetylmethylcarbinol reductase, the latter enzyme always being present in extracts from cells grown on 2,3-butanediol (Juni and Heym, 1956*a*). The methods used by Aubert and Millet (1953) would have failed to differentiate between acetylbutanediol and acetylmethylcarbinol and could account for the results they reported.

General properties of the system. The production of diacetylmethylcarbinol is linear with time until the DAMC concentration approaches 1.5×10^{-3} M, at which time the rate of its formation begins to fall off (figure 1). To rule out the possibility that diacetyl inactivates the enzyme slowly with time the points of the curve of figure 1 were also obtained by diluting the enzyme 1:3 and incubating each sample three times longer. The values obtained are virtually identical to those for shorter contact with diacetyl, thus proving that prolonging the exposure time to diacetyl with a diluted enzyme does not result in inactivation. These data also illustrate that the rate of the reaction is a linear function of the amount of enzyme employed. This last fact has also been verified in independent experiments where the enzyme concentration was varied over a five-fold range.

A typical curve for acetic acid production as a function of time may be seen in figure 2. The rapid departure from linearity is due to end product inhibition and is very similar to the result obtained for the decarboxylation of pyruvic acid by wheat germ carboxylase (Singer and Pensky, 1952). When diacetylmethylcarbinol was added initially, together with diacetyl, the reaction was considerably inhibited but proceeded linearly with time (figure 2) since the small amount of diacetylmethylcarbinol formed during the course of the experiment did not essentially alter the concentration of this end product in the reaction vessel. The influence of diacetylmethylcarbinol concentration on the initial rate of the reaction is illustrated in figure 3.

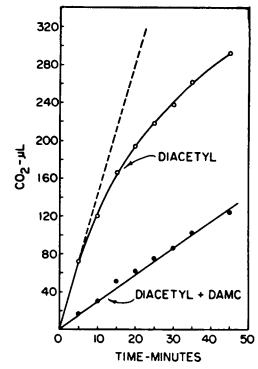

Figure 2. Acetic acid formation from diacetyl as a function of time in the presence and absence of diacetylmethylcarbinol. Standard conditions for the manometric assay were used. Diacetyl, 50 μmoles; diacetylmethylcarbinol, 34 μmoles; final vol 1.6 ml.

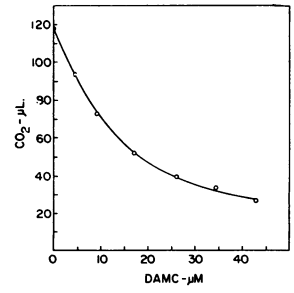

Figure 3. Inhibition of the initial rate of diacetyl dissimilation as a function of the concentration of diacetylmethylcarbinol. Standard conditions for the manometric assay were used. Diacetyl, 50 μmoles; final vol, 1.6 ml. The values for CO_2 evolved from bicarbonate buffer were obtained after incubation for 20 min in the presence of the indicated amounts of diacetylmethylcarbinol.

TABLE 2

Summary of purification procedure for diacetylmethylcarbinol-forming enzyme

Fraction	Volume	Protein	Activity	Specific Activity
	ml	*mg per ml*		
Crude	222	20.2	11.3	0.56
Amphojel supernatant	235	14.5	13.2	0.91
Protamine supernatant	250	13.3	11.7	0.88
C γ eluate	175	5.8	9.6	1.6
1st $(NH_4)_2SO_4$ precipitate	5.3	6.8	130	19.1

A diacetylmethylcarbinol concentration of 8.8×10^{-3} M inhibited the rate of the reaction by 50 per cent.

Partial purification of the enzyme system. During all the steps of the fractionation enzyme solutions were kept at 0–5 C. To 222 ml of crude sonic extract were added 55 ml of Amphojel (81.3 mg dry weight per ml) and the suspension stirred for 10 min. The gel was removed by centrifugation for 10 min at 25,000 × G and discarded. A fairly extensive study of this enzyme system showed it to be refractory to all the classical protein purification procedures until it was first treated with Amphojel or some other gel such as alumina C γ. The results in table 2 show that this treatment also resulted in a slight increase in activity due presumably to the removal of interfering substances.

Nucleic acid was removed by the dropwise addition, with continuous stirring, of 19.6 ml of a 2.0 per cent protamine sulfate solution, which had been adjusted to pH 6.4, to 235 ml of the supernatant obtained after the Amphojel treatment. The turbid suspension was stirred for 20 min and clarified by centrifugation for 20 min. The active protein was then adsorbed by first adjusting the supernatant after protamine treatment (250 ml) to pH 6.0 with 2.0 M sodium acetate buffer, pH 4.7, and then adding 175 ml of alumina C γ (27.8 mg dry weight per ml) slowly with stirring. After mixing for 10 min the gel suspension was centrifuged for 10 min and the supernatant fluid discarded.

The C γ gel was eluted with 175 ml of 30 per cent saturated ammonium sulfate solution (diluted from the following saturated solution: 70 g of $(NH_4)_2SO_4$ dissolved in 100 ml of water and adjusted to pH 7.3 with NH_4OH—the pH was measured with a sample diluted 1:50), the gel being thoroughly suspended with the aid of a cold mortar and pestle. The gel was removed by centrifugation for 10 min and the precipitated material discarded.

The ammonium sulfate eluate was found to remain stable when stored at −18 C for several weeks. To 175 ml of eluate were added 35 ml of saturated ammonium sulfate solution (pH 7.3) with continuous stirring. The slightly turbid suspension was centrifuged and the precipitate dissolved with distilled water to a final volume of 5.3 ml. This last fraction contained 27.8 per cent of the activity of the crude extract with an over-all purification of 34-fold. Storage of the purest fraction at −18 C overnight resulted in the loss of approximately 50 per cent of the activity. No attempt was made to stabilize the enzyme, since it is so readily derived from the stable ammonium sulfate eluate when required. It does seem likely, however, that reducing agents such as glutathione afford adequate protection since dialysis overnight against 0.025 M veronal buffer, pH 7.6, containing 0.01 per cent glutathione, resulted in the recovery of nearly all of the original activity in the fraction used.

Cofactor requirements. The activities of purified enzyme fractions can be stimulated several-fold by diphosphothiamin and magnesium ions. To obtain a better resolution a sample of purified enzyme was dialyzed against 0.6 per cent disodium dihydrogen ethylenediaminetetraacetate dihydrate, adjusted to pH 7.5 with sodium hydroxide for 12 hr at 5 C. The cofactor requirements for this dialyzed fraction are shown in

TABLE 3

Cofactor requirements for diacetylmethylcarbinol formation

Additions	Diacetylmethylcarbinol
	μmoles
Complete*	0.148
− DPT	0.014
− Mg^{++}	0.051
− DPT, − Mg^{++}	0.011

* Complete system: diacetyl, 5 μmoles; diphosphothiamin, 0.03 μmoles; $MgSO_4$, 1.2 μmoles; sodium maleate buffer, pH 6.4, 50 μmoles; enzyme, 0.01 ml; final vol, 0.5 ml; gas phase, air. Temperature, 30 C. Time of incubation, 10 min.

TABLE 4

Effect of divalent cations on the activity of diacetylmethylcarbinol-forming enzyme

Cation	Per Cent of Maximum Activity
Mg^{++}	100
Co^{++}	96
Mn^{++}	86
Ni^{++}	82
Ca^{++}	64
Cd^{++}	50
none	38

Assay system: diacetyl, 5 μmoles, diphosphothiamin, 0.03 μmoles; divalent cation, 8 μmoles; sodium maleate buffer, pH 6.4, 50 μmoles; enzyme 0.01 ml; final vol, 0.5 ml; gas phase, air; temperature, 30 C; time of incubation, 10 min.

table 3. Other divalent cations also activate the dialyzed preparation (table 4). Of all the cations tested some stimulation was observed in every case except for ferrous ions where an inhibition of 30 per cent of the basal activity (no cations added) was observed. Singer and Pensky (1952) report that ferric ions inhibit the basal activity of wheat germ carboxylase, and it is possible that the observed inhibition of the diacetylmethylcarbinol synthesizing enzyme may be due to the presence of ferric ions in the ferrous sulfate solution employed.

The Michaelis-Menten constant for diphosphothiamin was found to be 1.1×10^{-6} M. The comparable K_m for diphosphothiamin for the diacetyl enzyme from *N. winogradskyi* was reported to be 1×10^{-6} M (Aubert and Millet, 1953), while the K_m for the α-acetolactic acid forming system from *Streptococcus faecalis* was found to be 1×10^{-6} M (Dolin and Gunsalus, 1951).

Effects of pH. Unlike most diphosphothiamin enzymes the diacetylmethylcarbinol synthesizing system has a broad pH optimum from pH 6.0 to 8.0. Caution must be observed, however, since diacetyl dimerizes in alkaline solutions (Diels *et al.*, 1914). The curves in figure 4 show the disappearance of unpolymerized diacetyl as a function of the pH of incubation. It is apparent that some diacetyl dimerizes even at pH values less than 7.0; above pH 6.5 dimerization is very rapid. It is important to be aware of this behavior of diacetyl especially when attempting to do balance studies with this substrate. The experiments reported in this paper were all performed at pH 6.0 to 6.4 and 30 C.

Inhibitors. The various inhibitors of this reaction tested are listed in table 5. The enzyme does appear to contain sulfhydryl groups, since it is strongly inhibited by heavy metals and by *p*-chloromercuribenzoic acid and iodoacetic acid. The *p*-chloromercuribenzoic acid reagent is not as potent an inhibitor in this system as it is for the pyruvic acid carboxylases from *Acetobacter suboxydans* (King and Cheldelin, 1954) and from wheat germ (Singer and Pensky, 1952). Diacetylmethylcarbinol, the end product of the reaction, is also a fairly strong inhibitor of the system as shown in figure 3. The structural similarity of diacetyl and pyruvamide probably accounts for the inhibitory action of the latter compound.

Reversibility of the reaction. When purified enzyme preparations react with diacetyl very nearly all of the substrate is converted to diacetylmethylcarbinol and acetic acid. This indicates that the equilibrium of the reaction is in the direction of acyloin synthesis. To test for the possible reversibility of this reaction diacetylmethylcarbinol (25 μmoles) and sodium acetate (25 μmoles) were incubated with 0.5 ml of an active enzyme, together with the same amounts of the cofactors used to assay for the forward reaction, for 1 hour (reaction volume, 1.6 ml). No net synthesis of diacetyl could be detected using the sensitive method of Prill and Hammer (1938). It would thus appear that this reaction

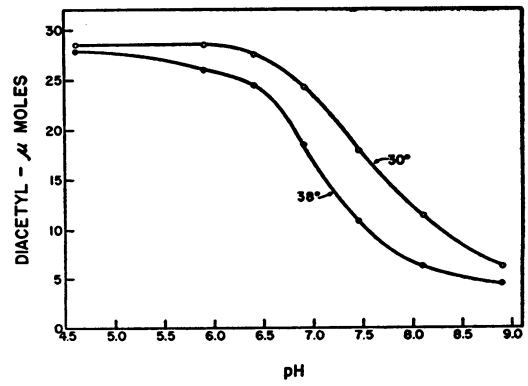

Figure 4. Dimerization of diacetyl as a function of pH. Each vessel contained 140 μmoles of potassium phosphate buffer at the pH indicated, 28.5 μmoles of diacetyl for the 30 C curve and 27.9 μmoles of diacetyl for the 38 C curve. Total vol, 1.6 ml. Incubation in 100 per cent nitrogen for 110 min.

is also similar to the analogous α-acetolactic acid forming system in that reversibility cannot be demonstrated in either case. To date, the only diphosphothiamin catalyzed reaction that has been shown to be freely reversible is transketolase (Horecker *et al.*, 1953).

Substrate specificity. Since the formation of diacetylmethylcarbinol from diacetyl is completely analogous to the synthesis of α-acetolactic acid from pyruvic acid (Juni and Heym, 1956*a*, *b*) the possibility that the same enzyme might catalyze both reactions was investigated. Pyruvic acid was found to be inert in the diacetyl system, and diacetyl is not a substrate for the α-acetolactic acid forming enzymes. Analogues of diacetyl such as acetyl isobutyryl and acetyl benzoyl were found to substitute for diacetyl in both the manometric and colorimetric assays. The nature of the products obtained with these analogues is now being investigated and will be the subject of a future report. The Michaelis-Menten constant for diacetyl is 6.3×10^{-4} M.

Evidence against the possible direct oxidation of diacetyl. The cyclic mechanism for the oxidation of 2,3-butanediol (Juni and Heym, 1956*a*) involves the acyloin condensation of diacetyl to diacetylmethylcarbinol as outlined above. Since the cyclic pathway was elucidated primarily from anaerobic studies there is the possibility that diacetyl may also be directly oxidized under aerobic conditions as well as undergo an acyloin condensation. The concept that both of these reactions may be catalyzed by a single enzyme is not an unlikely one, since it has been shown (Schweet *et al.*, 1951; Juni and Heym, 1956*b*) that pyruvic acid oxidase can oxidize its substrate aerobically or give rise to acyloin condensation products when oxygen or a suitable electron acceptor is absent. For the case of the diacetylmethylcarbinol system, however, it was shown that extracts active anaerobically on diacetyl will not oxidize diacetyl either in air or anaerobically in the presence of ferricyanide. Furthermore, a particulate cytochrome containing fraction[3] obtained from *Proteus vulgaris*, which can couple the oxidation of pyruvic acid to molecular oxygen (Moyed and O'Kane, 1956), did not serve to activate the diacetylmethylcarbinol forming system for oxygen uptake. The accumulation of acetylbutanediol in the medium when cells are grown aerobically on acetylmethylcarbinol (Juni and Heym, 1956*a*) may be taken as further evidence that a considerable portion, if not all, of the diacetyl metabolized condenses to form diacetylmethylcarbinol and acetic acid.

[3] Kindly supplied by W. E. Razzell.

TABLE 5

Inhibitors of diacetylmethylcarbinol-forming enzyme

Inhibitor	Concentration	Per Cent Inhibition
	molarity	
$HgCl_2$	2×10^{-5}	16
$HgCl_2$	2×10^{-4}	100
$AgNO_3$	8×10^{-6}	41
$AgNO_3$	4×10^{-5}	100
$CuSO_4$	4×10^{-4}	51
$CuSO_4$	4×10^{-3}	100
p-Chloromercuribenzoic acid	4×10^{-3}	88
Iodoacetic acid	7×10^{-3}	67
Pyruvamide	1×10^{-1}	86
Pyruvamide	2×10^{-2}	62
Acetaldehyde	2×10^{-1}	95
Acetaldehyde	4×10^{-2}	69
Diacetyl monoxime	4×10^{-2}	65
Acetylmethylcarbinol	1×10^{-2}	52
Methyl ethyl ketone	2×10^{-1}	55
Sodium arsenite	2×10^{-2}	51
Sodium pyruvate	2×10^{-1}	35
Sodium acetate	2×10^{-1}	15
Sodium fluoride	5×10^{-3}	13
Acetamide	2×10^{-1}	17

Artifacts encountered using diacetyl as a substrate. Diacetyl is a fairly reactive compound and can give rise to many artifacts when used as a substrate in biological systems. The volatility of diacetyl makes it necessary to employ suitable gassing controls when using an atmosphere other than air in the conventional Warburg vessel. Solutions of diacetyl slowly decrease in strength even when stored in the refrigerator and should be checked periodically or, preferably, made up before each experiment from freshly distilled diacetyl. Under even slightly alkaline conditions diacetyl can dimerize fairly extensively (figure 4) and experiments of long duration should be conducted at pH 6.0, or as low as possible. During enzyme fractionation it was noted that fairly strong solutions of ammonium sulfate react with diacetyl to give an acid in the manometric assay using bicarbonate buffer. The products formed in this non-enzymatic reaction have not yet been identified. Ammonium sulfate frac-

tions assayed during the purification procedure described above were not dialyzed before being tested because the high dilution used in the colorimetric assay did not introduce sufficient ammonium sulfate to cause any interference. When used in the large scale preparation of diacetylmethylcarbinol (Juni and Heym, 1957), however, ammonium sulfate fractions should be dialyzed before coming into contact with diacetyl. Tris buffer (tris-(hydroxymethyl)-aminomethane) also reacts rapidly with diacetyl and cannot be used in any system where diacetyl is the substrate.

DISCUSSION

For many years the only naturally occurring acyloin condensation product known was acetylmethylcarbinol. Recent studies (Juni, 1952*a*, *b*; Dolin and Gunsalus, 1951) have shown that there is only one enzyme system that appears to be uniquely concerned with acetylmethylcarbinol synthesis, namely the α-acetolactic acid forming and decarboxylating enzymes from bacteria that carry on a typical 2,3-butanediol fermentation of sugars. All other enzymes that form acetylmethylcarbinol do so as side reactions. In these cases the rates of acetylmethylcarbinol production are usually very small compared with the rates of the chief reactions of the enzymes (Juni, 1952b; Juni and Heym, 1956*b*). The discovery of transketolase (Horecker and Smyrniotis, 1953; Racker *et al.*, 1953), the enzyme concerned with the synthesis of 5 and 7 carbon sugars, revealed a second diphosphothiamin enzyme whose main function is acyloin synthesis. The diacetylmethylcarbinol forming enzyme, described in this paper, represents still another diphosphothiamin enzyme whose primary function is to catalyze the synthesis of a carbon to carbon bond containing adjacent carbonyl and carbinol groups (ketols). Pyruvic oxidase preparations also convert diacetyl, in part, to diacetylmethylcarbinol (Juni and Heym, 1956*b*) but this has been shown to occur as the result of a lack of substrate specificity of these systems and is probably of no physiological significance.

All acyloin condensations may be pictured as two stage reactions. The first step yielding an activated aldehyde which is condensed with an aldehyde or ketone in the second step. No evidence for the participation of two separate enzymes has been obtained during the fractionation of the diacetylmethylcarbinol forming system. Transketolase has been crystallized from baker's yeast (de la Haba and Racker, 1955) and only one enzyme seems to be concerned here with both the formation of the activated aldehyde ("active glycolaldehyde") and with its subsequent condensation to a suitable acceptor aldehyde. Strecker and Ochoa (1954), however, have presented evidence that two distinct enzyme fractions may be involved in the synthesis of α-acetolactic acid from pyruvic acid by *Aerobacter aerogenes*. Combination of these fractions resulted in a 2-fold increase over the sum of the activities of the fractions assayed separately. Unpublished studies from our laboratory on the purification of the α-acetolactic acid forming system from the same organism did not disclose any evidence for the participation of two enzymes in spite of the fact that over a 100-fold purification was achieved. The detailed mechanisms whereby diphosphothiamin serves to generate an aldehyde activated for acyloin condensation have yet to be elucidated.

One of the chief reasons for the great interest in the enzymatic synthesis of acetylmethylcarbinol was the fact that the reaction represents the formation of a carbon to carbon bond. This was thought by some to be a model for the synthesis of complex organic molecules from simple ones by living cells. It is only in recent years that the importance of acyloin condensations for the biosynthesis of sugars has served to focus attention once again on this reaction. The studies reported in this paper show that acyloin condensations play a role in the aerobic dissimilation of certain compounds by bacteria. Ketol condensation products have been postulated as possible intermediates in the synthesis of branched chain amino acids (Strassman *et al.*, 1955). It seems likely that as more acyloin condensations are discovered this reaction may assume a role of considerable more importance in the area of biosynthesis than was hitherto suspected.

SUMMARY

Cell-free extracts from bacteria grown in a medium containing 2,3-butanediol or acetylmethylcarbinol as the sole source of carbon and energy contain an enzyme that irreversibly con-

verts diacetyl to diacetylmethylcarbinol and acetic acid. The enzyme has been purified and the stoichiometry of the reaction studied. Diacetylmethylcarbinol is an acyloin condensation product, and diphosphothiamin and a divalent cation, such as magnesium, are the cofactors for the enzyme involved in its synthesis. Several inhibitors of the system have been studied. Diacetyl is not oxidized either aerobically or anaerobically in the presence of ferricyanide. The enzyme is specific for diacetyl and related alpha-diketones and will not degrade pyruvic acid. Diacetylmethylcarbinol is a fairly strong inhibitor for its own synthesis. Artifacts encountered when using diacetyl as a substrate have been discussed.

REFERENCES

AUBERT, J. P. AND MILLET, J. 1953 Dégradation du diacétyle par un extrait enzymatique bactérien. Compt. rend., **236**, 1512–1514.

DE LA HABA, G. AND RACKER, E. 1955 Crystalline transketolase from baker's yeast. *Methods in Enzymology I*, pp. 375–380. Academic Press Inc., New York.

DIELS, O., BLANCHARD, W. M., AND VON DER HEYDEN, H. 1914 Über Eigenschaften, Struktur, und Derivate des Dimeren Diacetyls. Ber. chem. Ges., **47**, 2355–2365.

DOLIN, M. I. AND GUNSALUS, I. C. 1951 Pyruvic acid metabolism. II. An acetoin-forming enzyme system in *Streptococcus faecalis*. J. Bacteriol., **62**, 199–214.

GREEN, D. E., STUMPF, P. K., AND ZARUNDAYA, K. 1947 Diacetyl mutase. J. Biol. Chem., **167**, 811–816.

HORECKER, B. L. AND SMYRNIOTIS, P. Z. 1953 The coenzyme function of thiamine pyrophosphate in pentose phosphate metabolism. J. Am. Chem. Soc., **75**, 1009–1010.

HORECKER, B. L., SMYRNIOTIS, P. Z., AND KLENOW, H. 1953 The formation of sedoheptulose phosphate from pentose phosphate. J. Biol. Chem., **205**, 661–682.

JUNI, E. 1952*a* Mechanisms of formation of acetoin by bacteria. J. Biol. Chem., **195**, 715–726.

JUNI, E. 1952*b* Mechanisms of formation of acetoin by yeast and mammalian tissue. J. Biol. Chem., **195**, 727–734.

JUNI, E. AND HEYM, G. A. 1956*a* A cyclic pathway for the bacterial dissimilation of 2,3-butanediol, acetylmethylcarbinol, and diacetyl. I. General aspects of the 2,3-butanediol cycle. J. Bacteriol., **71**, 425–432.

JUNI, E. AND HEYM, G. A. 1956*b* Acyloin condensation reactions of pyruvic oxidase. J. Biol. Chem., **218**, 365–378.

JUNI, E. AND HEYM, G. A. 1957 Preparation, properties and colorimetric determination of diacetylmethylcarbinol. Arch. Biochem. and Biophys., *Accepted for publication*.

KING, T. E. AND CHELDELIN, V. H. 1954 Pyruvic carboxylase of *Acetobacter suboxydans*. J. Biol. Chem., **208**, 821–831.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., **193**, 265–275.

MOYED, H. S. AND O'KANE, D. J. 1956 Enzymes and coenzymes of the pyruvate oxidase of Proteus. J. Biol. Chem., **218**, 831–840.

PRILL, E. A. AND HAMMER, B. W. 1938 A colorimetric method for the microdetermination of diacetyl. Iowa State Coll. J. Sci., **12**, 385–390.

RACKER, E., DE LA HABA, G., AND LEDER, I. G. 1953 Thiamine pyrophosphate, a coenzyme of transketolase. J. Am. Chem. Soc., **75**, 1010–1011.

SCHWEET, R. S., FULD, M., CHESLOCK, K., AND PAUL, M. H. 1951 Initial stages in pyruvate oxidation. *Phosphorus metabolism* Vol. I. The Johns Hopkins Press, 246–259.

SINGER, T. P. AND PENSKY, J. 1952 Isolation and properties of α-carboxylase of wheat germ. J. Biol. Chem. **196**, 375–388.

STADTMAN, E. R., NOVELLI, G. D., AND LIPMANN, F. 1951 Coenzyme A function in and acetyl transfer by the phosphotransacetylase system. J. Biol. Chem., **191**, 365–376.

STRASSMAN, M., THOMAS, A. J., AND WEINHOUSE, S. 1955 The biosynthesis of valine. J. Am. Chem. Soc., **77**, 1261–1265.

STRECKER, H. J. AND OCHOA, S. 1954 Pyruvate oxidation system and acetoin formation. J. Biol. Chem., **209**, 313–326.

WARBURG, O. AND CHRISTIAN, W. 1941 Isolierung und Kristallisation des Gärungsferments Enolase. Biochem. Z., **310**, 384–421.

WESTERFELD, W. W. 1945 A colorimetric determination of blood acetoin. J. Biol. Chem., **161**, 495–502.

WILLSTÄTTER, R. AND KRAUT, H. 1923 Über ein Tonerde-Gel von der Formel $Al(OH)_3$. II. Mitteilung über Hydrate und Hydrogele. Ber. chem. Ges., **56**, 1117–1121.